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REGENERATION OF COMPLETE HYDRA FROM ISOLATED EPIDERMAL EXPLANTS [1]

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A number of workers have studied the regenerative properties of isolated cell layers from coelenterates. Gilchrist (1937) and Steinberg (1963) reported the ability of isolated epidermis from *Aurelia* scyphistomae to regenerate into complete polyps. Zwilling (1963) found that isolated pieces of epidermis in *Cordylophora* regenerated into complete polyps but that isolated gastrodermis disintegrated. Although Papenfuss and Bokenham (1939) reported that neither cell layer in *Hydra* would regenerate, later work has shown that the gastrodermis of both brown and green hydra can regenerate whole animals. Normandin (1960) reported complete polyp regeneration from gastrodermal explants of *Hydra oligactis*, and Haynes and Burnett (1963) obtained similar regeneration from isolated gastrodermis of *Hydra viridis*.

However, regeneration of complete hydras both from isolated epidermis and from isolated gastrodermis of the same species has not been reported previously. The present paper reports the development of gastrodermis, and finally whole animals, from epidermal explants of *Hydra oligactis* and *Hydra pseudoligactis*, demonstrating the totipotency of each cell layer in *Hydra*.

The individual cell layers must be separated in order to test their regenerative potential. Various methods have been used to do this. Early workers relied on mechanical or surgical techniques, scraping or cutting away the gastrodermis. In *Aurelia* scyphistomae manual dissection is effective because a thick layer of mesoglea separates the cell layers. Papenfuss (1934) obtained endoderm-free cylinders of epidermis by scraping away gastrodermal cells from everted hydra, a laborious process. More recently, chemical or combination chemical and mechanical methods have been employed. Normandin (1960) removed the hypostome and tentacles and treated the body column with pancreatin and amylase to digest the mesoglea. Haynes and Burnett (1963) used a trypsin solution. Also in Burnett's laboratory Muscatine's solution (1961) brought to pH 2.5 with HCl has been used to remove the epidermis (Lenhoff and Muscatine, 1963). The acid and enzyme treatments, however, invariably damage the epidermal cells and no epidermal explants have shown any regenerative ability after such treatment.

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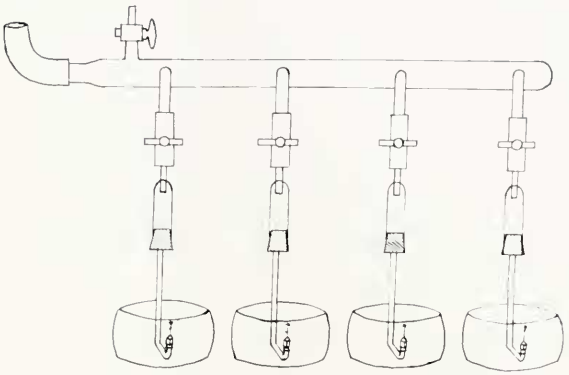


FIGURE 1. Perfusion apparatus (see text for explanation).

The present study of epidermal regeneration uses a simple perfusion technique which separates the cell layers cleanly without apparent injury to either epidermis or gastrodermis.

METHODS

Separation of epidermis and gastrodermis is accomplished by perfusing the gastrovascular cavity with Haynes' solution (0.2% NaCl, 0.2% $CaCl_2$, 0.03% $MgSO_4$, and 0.01% $KHPO_4$ made up in glass distilled water).

The perfusion fluid is contained in a reservoir supplying a glass manifold to which the individual perfusion units are attached (Fig. 1). Each unit has an

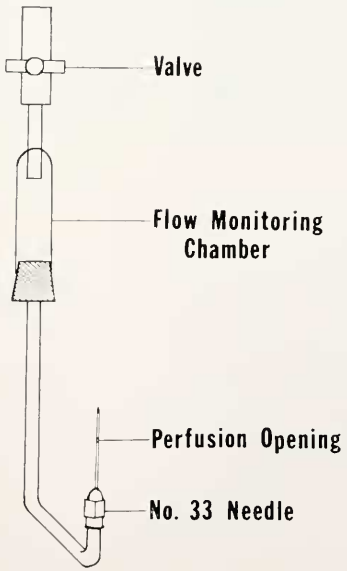


FIGURE 2. Perfusion unit.

adjustable clamp for admitting the perfusate, a transparent chamber for monitoring flow rate, and a J-shaped piece of glass tubing which carries a specially perforated #33 hypodermic needle on its lower end (Fig. 2). A hole is made in the side of the needle with a fine grinding wheel about $\frac{1}{3}$ inch from the tip and the tip sealed with epoxy (Hysol 1C White).

The hydra is threaded on the needle with the point entering the pore in the basal disc and coming out the mouth and the animal positioned so that the opening lies in the gastrovascular cavity. Since the hydra often works its way off the needle it is maintained in position by forcing a small piece of Parafilm over the needle tip (Figs. 3 and 4). The needle together with the lower part of the perfusion unit is immersed in a dish of standard culture medium (Loomis and Lenhoff, 1956) made up with distilled water. It is not necessary to maintain a specific rate of flow; the purpose of the monitoring chamber is to insure that flow does not stop from contraction of the animal around the needle.

The temperature of the perfusion dishes was maintained between 10–15° C throughout the separation process.

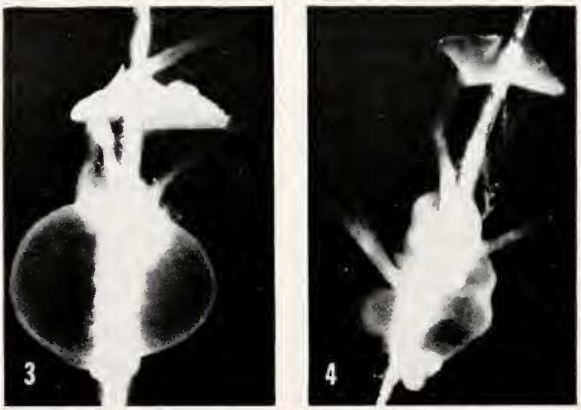


FIGURE 3. Hydra on perfusion needle showing epidermal separation. × 66.
FIGURE 4. Hydra on perfusion needle showing spontaneous eversion. × 66.

Separation is usually evident without the use of a microscope. Following separation the lower part of the perfusion unit, still immersed in its dish of culture medium and with the hydra still on the needle, is detached from the monitoring chamber and mounted in a clamp under a dissecting microscope. Iridectomy scissors and No. 5 watchmaker forceps are used for removing the epidermis. Once punctured, the separated epidermis tends to collapse inward, but by grasping the cut edge with the forceps it can be lifted away from the gastrodermis. Then by exerting gentle tension the epidermis can be cut away, the animal meantime revolving on the needle. In this way it is possible to obtain large pieces or cylinders of epidermis completely free from gastrodermis.

This technique for cell layer separation was tested on three species of brown *Hydra: H. fusca* (8 animals), *H. oligactis* (31 animals), and *H. pseudoligactis* (458 animals). It was also tested on *Hydra viridis* (18 animals). The brown

Hydra were taken from stock cultures maintained by the method of Loomis and Lenhoff (1956) with distilled water substituted for tap water, and the green *Hydra* were cultured by the same method but using commercially supplied spring water instead of tap water.

Regeneration studies were made on *H. fusca* (3 animals), *H. oligactis* (4 animals), and *H. pseudoligactis* (91 animals). Epidermal explants were cultured individually in small (6 cm) petri dishes in approximately 20 ml of standard culture medium (Loomis and Lenhoff, 1956), in standard culture medium containing 2½–3% Haynes' solution, and in two cases standard medium and Haynes' solution in equal proportions. Most of the cultures were kept at room temperature (20°–24° C), though a few were kept at 12° C.

Animals selected for histological study were fixed in Bouin's or glutaraldehyde, stained in toluidine blue at pH 8, dehydrated in alcohol, and mounted in permount.

Results

Cell layer separation

Separation of epidermis from gastrodermis by perfusion was successful in all species of *Hydra* tested. Frequency of separation was analyzed by species, temperature, nutrition and season. In the four species tested under a variety of conditions of nutrition, budding, and temperature, the separation frequencies (including all degrees of separation) were as follows: *H. fusca*, four separations out of eight animals tested; *H. oligactis*, eight separations out of 31 animals tested; *H. pseudoligactis*, 218 separations out of 458 animals tested; *H. viridis*, four separations out of eighteen animals tested.

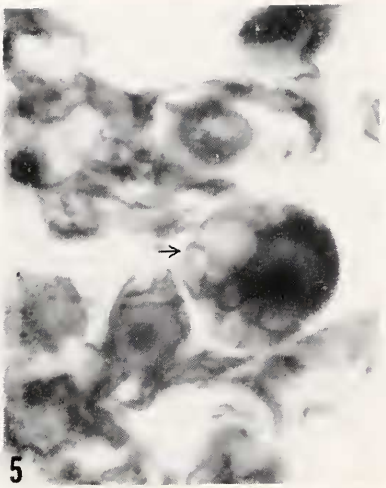
The effect of temperature on separation was tested with *H. pseudoligactis*. In a range from 5°–30° C the frequency of separation is inversely related to temperature. Between 25°–30° C one out of sixteen animals separated (6.25%). Between 15°–25° C 36 out of 100 animals separated (36%). Between 5°–15° C 173 out of 344 animals separated (50%). These figures include all degrees of separation.

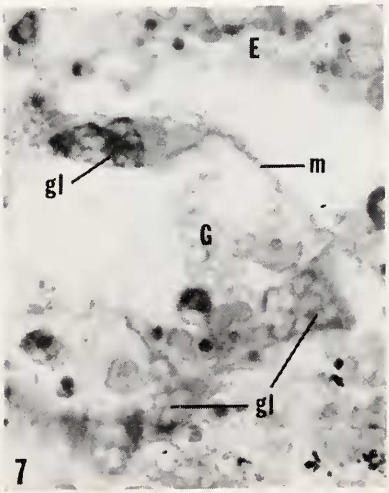
Separation frequency also changed seasonally. From late October through March, when this work was done, the frequency of separation increased through November and December to a maximum in January and February, then declined again in March.

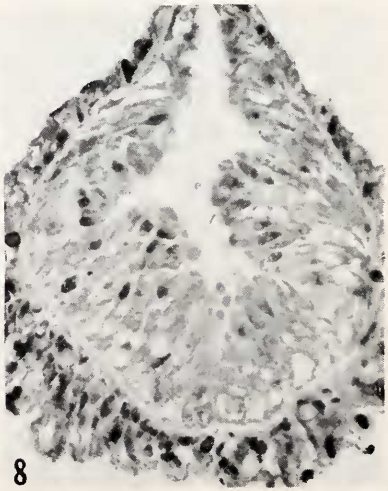
An additional factor correlated with separation frequency was nutrition. An analysis of this for *H. pseudoligactis* showed a separation frequency of 36% when perfusion occurred within 44 hours of feeding (177 animals), and 52% when perfusion occurred 45–100 hours after feeding (293 animals).

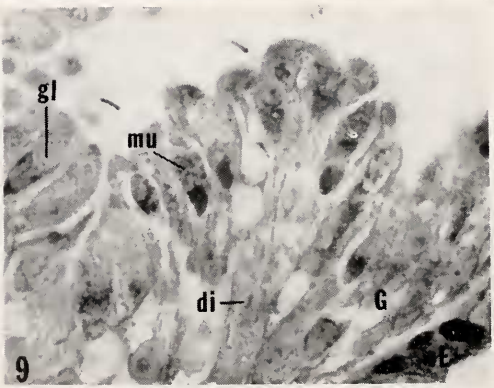
When the *Hydra* first accommodates itself to impalement the mouth usually closes around the needle so when perfusion begins the animal swells up like a miniature balloon. However, the mouth soon opens to establish an equilbrium.

Separation may occur at any time from five minutes up to two hours, with the majority of separations taking place within the first half hour. Separation may begin at any point between the tentacle ring and the basal disc. It proceeds as a wave proximally or distally or both from the point of origin, and also









FIGURES 5–9.

circumferentially often going completely around the animal. The result is the formation of a fluid-filled space between the inner and outer cell layers.

The locus of separation is in the mesoglea and lies closer to the gastrodermis than the epidermis. In stained sections of whole animals in which separation has occurred most of the mesoglea is seen attached to the epidermis. Separation of the mesoglea from gastrodermis can also be seen in the effect of continued perfusion of separated animals. After three to six hours of continued perfusion of a separated animal, tears occur in the epidermis exposing blisters of mesoglea. When this happens in a maximally separated animal the tears encircle the animal and the epidermis retracts or sloughs away, leaving behind a fluid-filled sac of naked mesoglea.

The extent of separation varies from a small blister of epidermis to maximum separation in which the entire epidermis between tentacles and basal disc forms a nearly transparent sphere in which the gastrodermis appears as a spindle contracted around the needle (Fig. 3). Ordinarily separation does not occur in the hypostome or tentacles or at the basal disc, and frequently only partial separation occurs in the peduncle. There is no separation around the projecting portion of a developing bud.

Occasionally a maximally separated animal will begin to evert on the needle. When this occurs the outrolling lip of the hypostome carries the tentacle ring and separated epidermis basally over the gastrodermis (Fig. 4), often detaching the epidermis of the basal disc from the gastrodermis. In such cases it is possible to cut below the tentacles and remove virtually the entire epidermis from the animal.

When a piece of epidermis is removed it curls up, scroll-like, along its proximo-distal axis, presumably due to contraction of the muscle fibers at the base of the epithelio-muscular cells. When an epidermal cylinder is removed it contracts to form a doughnut-like ring.

Regeneration of epidermal explants

A total of 98 epidermal explants were tested for regenerative ability. These differed in size and original location on the body column. Small explants, $\frac{1}{8}$ or less of the body column epidermis, invariably disintegrated within 12–48 hours. Larger pieces, up to entire epidermal cylinders, showed varying degrees of regeneration of a coelenteron and an inner gastrodermal cell layer in 17% of cultures, though regeneration was not a one-to-one function of the size of the explant. Frequency and degree of regeneration did not increase after the explant exceeded approximately one-half the total body column epidermis.

FIGURE 5. Stage I regenerate, *H. fusca*; note zymogen-like cell among epidermal cells (arrow), Toluidine blue, pH 8. × 2230.

FIGURE 6. Stage II regenerate, *H. pseudoligactis*; note flattened layer of gastrodermal cells lining coelenteron, Toluidin blue, pH 8. × 890.

FIGURE 7. Stage II regenerate, *H. pseudoligactis;* E, epidermis; G, gastrodermis; m, mesoglea; gl, gland cell; Toluidine blue, pH 8. × 910.

FIGURE 8. Stage III regenerate, *H. pseudoligactis*; note fully differentiated gastrodermis, Toluidine blue, pH 8. × 360.

FIGURE 9. Stage III regenerate, *H. pseudoligactis*, E, epidermis; G, gastrodermis; gl gland cell; mu, mucous cell; di, digestive cell; Toluidine blue, pH 8. × 910.

Because of the cleanness of separation between the cell layers and the efficiency with which the epidermis could be cut away, the problem of gastrodermal contamination of epidermal explants was eliminated. With practice it was possible to secure pieces of epidermis which were completely gastrodermis-free. As a check 10 epidermal pieces were fixed, sectioned, and stained immediately after removal. In no case were gastrodermal cells present.

Seventeen of the 98 explants observed showed varying degrees of regeneration. Each of the three species tested (*H. oligactis*, *H. pseudoligactis*, *H. fusca*) showed regenerative ability.

When regeneration occurs at room temperature visible changes appear in the first 24 hours. Morphogenesis continues actively during the second day, after which the rate of change declines over the next four or five days. For convenience in reference the events in regeneration have been grouped in stages as follows:

Stage I Cavitation Depending on the size of the explant, one or more small cavities become visible with a dissecting microscope within six to twenty hours. Examination of sectioned material fixed at six and twelve hours indicates that these cavities arise through the coalescence of intercellular spaces appearing in the first few hours. At the end of this stage there is no gastrodermal layer, although the cavities may show partial bordering by a mesoglea-like membrane, and scattered cells may be present inside the membrane as well as lying free within the cavity. Zymogen-like cells may be found occasionally near the cavity or along the recesses ramifying from it, (Fig. 5). During this stage the regenerate begins to show contractile movements.

Stage II Body column, hypostome, and tentacle formation Further morphogenesis depends to some extent on the body region from which the epidermis was taken. When the explant comes from the sub-hypostome and gastric regions (most commonly), a hypostome and/or tentacle buds are the next structures to appear. When the explant includes basal disc epidermis, the regenerate may form a basal disc instead of a hypostome. In three such cases no hypostome or tentacles formed at all, even though maintained in culture for two weeks. Concomitantly there is a lengthening of the regenerate as a body column is formed. During this period extension and fusion of the previous cavities occur forming a coelenteron. The mesogleal layer is completed between the epidermis and a developing gastrodermis (Fig. 6). Early gastrodermal cells appear on the inner surface of the mesoglea as a lining of flattened epithelium (Fig. 6), which later becomes thickened and contains presumptive gland and digestive cells (Fig. 7). In this stage there is also a noticeable increase in rate and extent of spontaneous contractions.

Stage III Mouth and basal disc formation With the appearance of a mouth the regenerate becomes a potentially independent organism. Two explants were followed through to this stage though both were too small to ingest *Artemia*. A basal disc developed in only one of these, one which contained a small amount of basal disc epidermis originally. Had the other regenerate been able to feed the development of a basal disc would presumably have occurred. Histologically these regenerates show completely organized epidermal and gastrodermal layers (Fig. 8), in which the gastrodermis contains fully differentiated gland, mucous, and digestive cells (Fig. 9).

Discussion

The epidermal cell type or types which give rise to the gastrodermal layer are not known. Nevertheless we believe it probable that interstitial cells play a prominent role in the process. Gastrodermal regeneration in *Hydra* may be analogous to gastrodermal regeneration in *Cordylophora* which Diehl (1969) has reported. He has followed regenerative stages in epidermal explants from *Cordylophora* in which interstitial cells move to the base of the epithelio-muscular cells soon after the epidermis is isolated. These cells become elongate, vacuolated, and differentiate directly into normal digestive cells. Paul Rose (personal communication) in our laboratory, also working with *Cordylophora*, has combined epidermis labeled with tritiated thymidine with the gastrodermis from a "cold" animal and studied the regeneration of new feeding hydranths at different time periods. He finds basophilic and secretory cells in the gastrodermis labeled, strongly implying that the labeled epidermal interstitial cells migrate to the interior of the regenerating explant and differentiate into the zymogen and mucous secretory cells.

Although our studies are in their early stages, we suspect that gastrodermal regeneration in *Hydra* will parallel that of *Cordylophora*. Different stages in the regenerative process are now being prepared for study with the electron microscope to determine ultrastructural changes in interstitial cells during the differentiation process.

Perhaps the major significance of the present study is that in *Hydra* each layer alone can give rise to the other during regeneration. Details of the regeneration of epidermis from gastrodermis have been worked out by Davis, Burnett, Haynes, and Mumaw (1966). The fact that these complementary regenerations occur in media of different ionic concentrations strengthens our belief that ionic environment is an important factor in the control of cell differentiation *in vivo* (Burnett, 1966; Macklin and Burnett, 1966).

As a variation of the epidermal regeneration described above, we have taken rings of isolated epidermis and threaded columns of isolated gastrodermis through them. In this way we have produced "composite" hydra which developed to mature feeding and budding individuals. In some cases the combinations were conspecific and in others interspecific, both types yielding successful regenerates.

Summary

1. A method of separating cell layers in *Hydra* by perfusion is presented. Under optimum conditions about 50% of perfused animals show separation of epidermis from gastrodermis. With this technique it is possible to secure large pieces and even whole cylinders of living epidermis or gastrodermis free from contamination by the other cell layer.

2. Isolated explants of epidermis were tested for regeneration in *H. fusca*, *H. oligactis*, and *H. pseudoligactis*. Each species showed some regenerative ability, and complete minature hydras were obtained in *H. oligactis* and *H. pseudoligactis*. Other work (Haynes and Burnett, 1963; Normandin, 1960) has shown that isolated gastrodermis can regenerate complete hydras in *H. viridis* and *H. oligactis*. Thus the present work, showing that isolated epidermis can also regenerate complete polyps, makes *Hydra* the first animal in which it has been demonstrated that each layer alone can give rise to the other during regeneration.

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